Functional Characterization of Arsenic Stress Tolerant Gene(s) in Azolla (*Azolla filiculoides* Lam.)

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List of abbreviations

AsV	Arsenate
AsIII	Arsenite
PCs	Phytochelatins
MTs	Metallothioneins
CPK31	Calcium Dependent Protein Kinases 31
MATE	Multidrug And Toxic Compound Extrusion
HAC1	High Arsenic Content 1
ACR3	Arsenic Compound Resistance 3
PPFD	Photosynthetic Photon Flux Density
RGR	Relative Growth Rate
RFSA	Relative Frond Surface Area
F_V	Variable Fluorescence
F_m	Maximum Fluorescence
CAT	Catalase
POD	Peroxidase
TCA	Trichloroacetic Acid
NBT	Nitroblue Tetrazolium
DAB	3, 3′-Diaminobenzidine
H2O2	Hydrogen Peroxide
AAS	Atomic Absorption Spectrometer
qRT PCR	Quantitative Real Time PCR
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
PCR	Polymerase Chain Reaction
FPKM	Fragments Per Kilobase of transcripts per Million fragments sequenced
FDR	False Discovery Rate

1. Abstract

This study investigates the effects of arsenic (As) stress on *Azolla filiculoides* to identify and characterize arsenic stress responsive genes. The relative growth rate (RGR) of *A. filiculoides* significantly decreased after six days of As exposure, with 91.4%, 71.4%, and 68.5% reductions observed at 10 20 and 30 μM As concentrations, respectively. Root traits, including length, number of roots, and root hairs, were negatively impacted by increased As levels, with root length decreasing by up to 57.3% at 50 μM As, although root diameter increased by 16.21% at 40 μM As. Photosynthetic performance, assessed through total pigment content and OJIP parameters, was also affected, showing a notable reduction in the Fv/Fm ratio under As stress. However, plants exposed to 20 μM As exhibited the lowest decrease in Fv/Fm (2.37%) compared to control plants. Interestingly, plants treated with 30 μM As showed no stress phenotypes and accumulated significant As (15 μg/g/Fw). Further, transcriptome analysis revealed upregulation of several key transporters, including ACR3, ABC transporter superfamily, and acetate uptake transporter (AceTr) family, under moderate As stress (10 μM). These transporters are crucial for vacuolar sequestration of As, suggesting an inherent As tolerance mechanism in *A. filiculoides*. However, transporters like CPA1, sugar transporters, and PiT were highly downregulated. Additionally, RNA-seq analysis under various As stresses (0, 10, and 20 μM) identified differentially expressed genes (DEGs) related to As stress, such as *ACR3*, which promotes As efflux from cells. Gene ontology (GO) and KEGG pathway enrichment analyses highlighted various associations of DEGs and pathways involved in As stress response. Notably, transcription factors like C2H2 finger domain and WRKY45 were differentially regulated. Further overexpression of identified *AfACR3* in transgenic *Arabidopsis thaliana* (Col-0) plants revealed improved arsenic tolerance; and sub-cellular localization study of GFP tagged ACR3 protein confirms that ACR3 is involved with arsenic efflux. These findings underscore *A. filiculoides*' potential for As phytoremediation and provide insights into its molecular response mechanisms, valuable for future crop improvement programs through genetic engineering.

2. Introduction

Arsenic is a toxic metalloid abundantly present in nature and vey toxic to plants, animal and humans (Podgorski and Berg, 2020) (Guha and Dasgupta, 2011; Milton et al., 2005; Smith et al., 2002; Taylor and Mukherjee, 2003). Prolong arsenic exposure is a leading cause for a number of diseases including arsenicosis, neurodegenerative diseases, cardiovascular diseases and cancer (Argos et al., 2010; Chen et al., 2011). The source of arsenic pollution is believed to be a non-point geogenic one, and majority of which contributed by arsenic contaminated aquifers. Consequently, though various efforts of avoiding have been taken to avoid the use of arsenic contaminated ground water , arsenic is still finding its way to human system by being accumulated in crop plants (Jian et al., 2008). So, understanding the process of accumulation, assimilation and remediation of arsenic is of grave importance. The natural predominant inorganic forms of arsenic are arsenate (AsV) and arsenite (AsIII); among these arsenate (AsV) is more bio-available due to its structural similarity with inorganic phosphate (Mitra et al., 2017; Tripathi et al., 2007). High-affinity phosphate transporters are the main transporters that take up arsenate (AsV) in plant system, on the other hand arsenite (AsIII) is mainly incorporated through aquaporins and other non-specific transporters (Srivastava et al., 2007). Upon entering the plant system a AsV is readily converted to AsIII by arsenate reductases enzyme; then a significant portion of AsIII is being chelated by phytochelatins (PCs), metallothiones (MTs) and sequestered inside vacuoles, remaining arsenic then translocated by various transporters to different part of the plant.(Biswas and Ganesan, 2024). However, arsenic tolerant organisms possess an efflux system where AsIII is directly extruded from the organism(Biswas and Ganesan, 2024). This arsenic efflux system is yet to be reported in angiosperms, have only been identified in a pteridophyte *Pteris vittata* which is terrestrial arsenic hyper accumulator plant (Indriolo et al., 2010).

Aquatic plants like *Eichhornia crassipe, Hydrilla verticillata, Potamogeton pectinatus* have been identified as arsenic hyperaccumulator (Kumar et al., 2015). Along with that, phytoremediation properties of *Azolla caroliniana* demonstrate significant promise in arsenic removal from contaminated water (Sufian et al., 2018). According to a recent study *Azolla* has been reported to uptake and withstand arsenic as well, among the other species, *Azolla filiculoides* Lam. has been reported to be the most resistant to Arsenic due to its lower As accumulation rate (Zhang et al., 2008). Moreover, *Azolla*'s convenient harvest and desiccation methods make it a perfect choice for phytoremediation of heavy metal like arsenic in

wastewater (Y Peng, 2007). As a result, *Azolla* stands as a promising bioremediation option for treating various types of wastewaters, including industrial effluents and sewage water. Due to the presence of symbiotic nitrogen fixing blue green algae in *Azolla*, intercropping with rice and *Azolla* are very useful for the farmers to reduce the nitrogen fertilizer application (Thapa and Poudel, 2021). Accelerated growth rate (doubling time and biomass production) and an extraordinary ability to accumulate toxic heavy metals (Cd, Cr, Ni, Zn and Cu) are the other notable characteristics of *Azolla* plants (Bennicelli et al., 2004; SELA et al., 1989).

The present work focuses on elucidating the phytoremediation properties like growth pattern, accumulation and tolerance of arsenic in *Azolla filiculoides*. Both As(V) and As(III) are readily available in the soil solution between pH 4 to 8 (Inskeep et al., 2002). Notably, *Azolla* can thrive between pH 3.5 to 10 (Adhikari et al., 2020) therefore, we selected *Azolla* plants for the current study. Along with that we have investigated the molecular response to arsenic stress in *A. filiculoides* plants at transcript level. We have identified several gene families involved with arsenic stress in *A. filiculoides*. In particular, the family of multidrug and toxic compound extrusion proteins (*MATE*) (Kuroda and Tsuchiya, 2009; Tiwari et al., 2014), calcium-dependent protein kinases (*CPK31*) (Ji et al., 2017), arsenate reductases like High Arsenic Content 1 (*HAC1*) and efflux transporters like Arsenic compound resistance 3 (*ACR3*) (Indriolo et al., 2010; Li et al., 2020; Shi et al., 2016) are very important. Hence, in this study, we evaluated the relative gene expression levels of *MATE1*, *CPK31*, *HAC1* and *ACR3*. We have reported the presence of *ACR3* in *Azolla* and we have developed transgenic *Arabidopsis thaliana* plant with *AfACR3* for functional characterization.

3. Objectives

Objective 1: Characterization of arsenic tolerance mechanism of *Azolla filiculoides* under different arsenic (As) stress conditions.

Objective 2: Identification of the arsenic stress-responsive gene(s) by transcriptome assay from *Azolla filiculoides* treated with different arsenic stress conditions.

Objective 3: Development of transgenic *Arabidopsis* plants with the key gene(s) which are responsible for arsenic stress-tolerance of *Azolla* plants.

4. Methodology

4.1. Plant material, growth conditions and arsenic treatment

Azolla filiculoides plants were collected from Accharya Jagadish Chandra Bose Indian Botanic Garden, Kolkata, India. Further, the plants were transferred to a *Azolla* pond (12x8 ft) which contains around 5 kg of fertile soil as a source of nutrition. The experiments were performed in the plant growth chamber (Weisstechnik®) at 25 ± 2 ºC and 60% relative humidity, operating on a 16/8 hrs photoperiod under 4400K white light at 100 µMol m^{-2} s^{-1} PPFD (Photosynthetic photon flux density). Plastic phytajars (SKU, PW1302, Himedia, India) were employed, each containing mature and healthy *Azolla* plants. At the beginning of the experiment 1g of fresh green plants were taken and cleaned with distilled water (dH_2O) and then transferred to each phytajar containing 300ml (pH 5) of IRRI2 meadia containing 40 µM $CaCl_2 \cdot 2H_2O$, 40 µM $MgSO_4 \cdot 7H_2O$, 40 µM K_2SO_4, 20 µM $NaH_2PO_4 \cdot H_2O$, 0.01 µM $CuSO_4 \cdot 5H_2O$, 0.2 µM H_3BO, 0.15 µM $Na_2MoO_4 \cdot 2H_2O$, 0.01 µM $ZnSO_4 \cdot 7H_2O$, 0.01 µM $CoCl_2 \cdot 6H_2O$, 0.5 µM $MnCl_2 \cdot 4H_2O$, 0.5 µM Fe-EDTA-Na_2 (Pereira and Carrapico, 2009). The experimental setup comprised six sets of phytajars for each treatment group: 0, 10, 20, 30, 40, and 50 µM sodium arsenate ($Na_3AsO_4 \cdot 12H_2O$), with each treatment replicated more than five times. After a cultivation period of 6 days, samples of *Azolla* were collected from each phytajar for analysis.

4.2. Morphological, physiological and biochemical attributes

4.2.1. Morphological attributes

Relative growth rate (RGR) of the control and stress treated plants were assessed by measuring the fresh weight of the plant (W_0) at the onset of the experiment and again measured after (6 days) of treatment (W_t), then the RGR was computed using the following formula (Jafarirad et al., 2019). $RGR = (\ln W_0 - \ln W_t)/t$ (t represents the time interval).

One of other important morphological change in response to arsenic stress is determined by the relative frond surface area (RFSA) (Paul et al., 2021) . To determine the RFSA images of plants were taken under uniform bright lighting at the beginning of the experiment and also after the arsenic treatment. Then the changes in the frond surface area was determined by analyzing the before and after images with Fiji-Image J software. Initial surface area of fronds at 0 days ($A0$) and final surface area (At where t = 6 days) at 6 days were measured. RFSA was calculated by the following formula; RFSA (%) = (At - $A0$)/ $A0$ ×

100. Root morphology of the plants after 6 days of arsenic treatment was measured under a light microscope and image taken with appropriate scale. Then root diameter and root hair length was measured using Fiji-Image J software.

4.2.2. Extraction and estimation of photosynthetic pigments

Chlorophyll estimation was done by homogenizing the plant materials (1g) in 80% acetone and centrifuged in 10000rpm for 10mins. The absorbance of the supernatant was taken at 470nm, 647nm and 663nm using 80% acetone as blank. To calculate the amount of chlorophylls and carotenoids, the following formula was used (Lichtenthaler, 1987).

Chlorophyll a= (12.25×A663) - (2.79×A647) μg/mL
Chlorophyll b= (21.5×A647) - (5.1×A663) μg/mL
Total carotenoid= [(1000×A470) - (1.82×Chla) - (85.02×Chlb)]/198 μg/mL

4.2.3. Estimation of chlorophyll fluorescence

For the evaluation of chlorophyll fluorescence, control and arsenic stress treated plants were subjected to 1-hour dark adaptation and then measurements were taken with a pocket PEA (Plant Efficiency Analyser, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) under dark condition. For the determination of fluorescence induction an actinic light of 3500 $\mu molm^{-2}s^{-1}$ was used with peak wavelength of 627 nm. The minimum fluorescence (F_0) to maximum fluorescence (F_m) (F_t, fluorescence at time t after actinic illumination; F_0 = 50ms; $F_J = F_{2ms}$, J step fluorescence intensity; F_I = I step fluorescence intensity; $F_P = F_m$, maximum fluorescence at peak P of OJIP) of the sampled plants were obtained from the pocket PEA data and analyzed trough PEA Plus version 1.13 software (Hansatech Instruments Ltd., King's Lynn, Norfolk, UK). All the parameters related to PSII photochemistry of OJIP transient were evaluated by the method described by Strasser et al. (Strasser et al., 2010).

4.2.4. Extraction and estimation of proline

Proline extraction and estimation was done by following the method, where 1g plant tissue was homogenized in 2mL 3% sulphosalycylic acid and centrifuged at 10000 rcf for 10 min at room temperature and then supernatant was collected. 2mL supernatant was mixed with 2mL acetic acid and 2mL 1% acid ninhydrin in a glass-tube and incubated in 96ºC for 60 min. After that 4mL toluene was added to the solution and vortexed for phase separation

of the choromophore, and then the absorbance of the chromophore was taken at 520nm and estimated against L-proline calibration curve (Abraham and Dhar, 2010).

4.2.5. Estimation of anti-oxidative enzymes activity

Extraction of anti-oxidative enzymes from the control plants and arsenic treated plants was done by pulverizing the freshly weighed plant material in liquid nitrogen and then the homogenized in 1.5mL of 50mM sodium phosphate buffer of pH 7.0 containing β-mercaptoethanol, 4% PVP-40 and 2mM EDTA in ice cold condition. Then centrifuged at 30000 rcf for 30min at 4ºC and supernatant was collected for further work. Peroxidase (POD) (EC: 1.11.1.7) activity was measured by using the reaction mixture consisted of Sodium phosphate buffer (100 mM, pH 7.0), 10 mM H_2O_2 solution, 20 mM guaiacol and enzyme extract. The reaction was started by adding guaiacol (20 mM) (Prasad and Singh, 2011). The change in the absorbance was recorded for 3min at 1min interval at 470nm, while the reaction mixture without the enzyme extract was served as blank. Further, catalase (CAT) (EC: 1.11.1.6) activity was measured By using the reaction mixture consisted of Sodium phosphate buffer (100 mM, pH 7.0), 30 mM H_2O_2 solution and enzyme extract (Yadav et al., 2016). The reaction was started by adding H_2O_2 solution and changes in absorbance was taken at 240nm for 1min at 15sec interval. The reaction mixture without the H_2O_2 solution was served as blank.

4.2.6. Estimation of Hydrogen peroxide (H_2O_2) content

For the estimation of H_2O_2 content, *Azolla* plants (1.0 g) was homogenized in 3mL 1% trichloroacetic acid (TCA) and centrifuged at 10000rcf for 10min at 4ºC and supernatant was collected. Reaction mixture was consisted of 0.75 mL potassium phosphate buffer (10 mM, pH 7.0), 1.5 mL 1M potassium iodide (KI), 0.75 mL of extract and the absorbance was taken at 390 nm. The H_2O_2 concentration was measured by comparing with a H_2O_2 calibration curve (Ghosh et al., 2011).

4.2.7. Histochemical detection of superoxide radicals and hydrogen peroxide

Nitroblue tetrazolium (NBT) staining was used for the visualization of superoxide radicals inside the plant tissues by the method of Grellet Bournonville & Díaz-Ricci, 2011 (Grellet Bournonville and Díaz-Ricci, 2011) with minor modifications. 0.2% NBT was dissolved in 50mM sodium phosphate buffer (pH 7.5) in a dark condition for the staining. *Azolla* plants were collected after 6days of various arsenic stress treatments, washed thoroughly with distilled water and kept in the staining solution in dark condition overnight.

For chlorophyll removal the plants were kept in ethanol at 65ºC for 10mins. After that kept in filter paper soaked with 60% glycerol solution and finally pictures were taken with a compound light microscope. For the detection of *in situ* peroxide (H_2O_2) production, 3, 3′-diaminobenzidine (DAB) staining was used (Liu et al., 2012) with slight modification. 1mg/ml DAB was dissolved in distilled water of pH 3.6 using 0.1N HCl. *Azolla* plants were placed in the staining solution overnight and followed by the chlorophyll removal by ethanol treatment at 65ºC for 10mins. Then plants were kept in 60% glycerol-soaked filter paper for further analysis under compound light microscope.

4.3. Atomic absorption measurement of arsenic accumulation

Arsenic content of *Azolla* tissue was determined by atomic absorption analysis after 6d treatment in different concentration of arsenic. Freshly collected samples were dried at 70ºC for 12 hrs and then completely dried by liquid nitrogen. Then 75% nitric acid was added and incubated at 90ºC for 2h followed by centrifugation at 13000g and filtered through filter paper. After that all samples are diluted in distilled water and measurements were taken using Atomic Absorption Spectrometer (AAS) (Shimadzu®, AA6501).

4.4. Expression analysis of arsenic responsive genes

Primers for the study were specifically designed for *MATE1*, *CPK31*, *HAC1* and *ACR3* gene based on the data obtained from previous existing literature and FernBase database (https://fernbase.org/) (Indriolo et al., 2010; Ji et al., 2017; Li et al., 2018; Wang et al., 2018). The coding sequences of these genes were obtained from the previously mentioned database by using BLAST tool. For the total RNA extraction, 100mg of plant samples was used and total RNA was isolated using NucleoSpin® RNA plant and fungi kit (Macherey-Nagel). The quality and quantity of isolated total RNA were measured by Micro-volume Spectrophotometer (Eppendorf BioPhotometer™ D30). Further, 500ng RNA for each treatment; first-stand cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara®, Japan). The qRT-PCR was performed using non-skirted 96-well PCR plate in a CFX Duet Real-Time PCR System (BIO-RAD) using β-*actin* as the reference gene. The reaction was performed with three biological and three technical repeats of 10µL reaction mix using 5µL of 2x iTaq Universal SYBR Green Supermix (BIO-RAD). The thermocycling condition was started with initial denaturation at 95ºC for 30sec followed by 39 cycles at 95ºC for 3sec, 59ºC for 20sec and 65ºC for 5sec with a final step of 95ºC for 15sec. The

relative fold change in gene expression was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

4.5. Transcriptome analysis

4.5.1. RNA extraction, cDNA library preparation, and sequencing

Total RNA was extracted from the arsenic treated plants through the previously mentioned procedure in 3.4. The quality and quantity of isolated total RNA were measured by Micro-volume Spectrophotometer (Eppendorf BioPhotometer™ D30). One microgram of RNA was employed as input material for RNA sample preparation and sequencing library generation by using NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® (NEB, MA, USA). Then,using poly-T oligo attached magnetic beads were used for the mRNA purification in RNA Binding Buffer (2X) (NEB). First strand cDNA synthesis reaction was done using NEB Next First Strand Synthesis Enzyme Mix, followed by assembling the second strand cDNA synthesis using NEBNext Second Strand Synthesis Enzyme Mix. Then second strand cDNA synthesis product was subjected to NEBNext Adaptor ligation and purification. Adaptor ligated DNA fragments then subjected to PCR enrichment using NEBNext Ultra II Q5 Master Mix and Universal PCR Primer/i5 Primer in a 15 cycle PCR reaction. The PCR product was purified using NEBNext Sample Purification Beads and then subjected to quantification and quality assessment using high sensitivity d1000 screentape® in Agilent 4200 TapeStation bioanalyzer system. The library was then sequenced on Illumina Novaseq 6000 sequencing platform (ABA Biotech, India).

4.5.2. Raw data processing, *de-novo* Transcriptome assembly generation and clustering

Before assembly and clustering the raw data generated through sequencing has to be filtered to assemble, for this purpose adapter trimming and quality trimming at Q30 was performed using Adapter Removal ver. 2.3.2 (Schubert et al., 2016). This program searches for and removes remnant adapter sequences from your read data. The program can analyze both single end, paired end data and merge overlapping pair ended reads into longer consensus sequencing, which gives us the clean read data in FASTQ format for further analysis. This clean read data was used for the *de novo* transcriptome assembly generation using Trinity version: v2.1.1 (Grabherr et al., 2011). During this process the data was assembled into the unique sequences of transcripts, isoforms and then to unique portions of alternatively spliced transcripts. Then the contigs transformed into clusters, each one

representing a full transcriptional complexity of a gene. The Trinity transcriptome assembly was further considered for clustering using CD-HIT version 4.7 (Fu et al., 2012). Cluster database at high identity with tolerance (CD-HIT) was used specifically produce non-redundant representative sequence and reduce the data size without losing any sequence for further analysis.

4.5.3. Expression estimation and differential transcript analysis

The obtained data was subjected to expression estimation analysis using kallisto 0.46.2 (Bray et al., 2016). Using this program the quantification of abundances of transcripts from the sequencing data was done to analyses the expression of a given transcript. Fragments PerKilobase of transcripts per Million fragments sequenced (FPKM) value of the transcripts was calculated to determine the expression level (Trapnell et al., 2010). Then the differential gene expression analysis was performed using edgeR version 3.42.4 (Robinson et al., 2010). This process is concerned with the differential expression by analyzing the expression of replicated count data of the transcripts. Transcripts with false discovery rate (FDR) < 0.01, p-value < 0.05 and $\log_2$Fold-Change ≥ 1 or ≤ -1 were assigned as differentially expressed genes (DEGs). The differential expression data of most variably expressed DEGs were used for hierarchical cluster generation and principle component analysis in iDEP (Ge et al., 2018) and later annotated to see the relationship among highly expressed DEGs and their expression pattern in response different arsenic stress.

4.5.4. Annotation, identification of DEGs and transcription factor analysis

The clustered transcripts were subjected to homology search against uniprot database using diamond v2.0.8.146 (Hernández-Salmerón and Moreno-Hagelsieb, 2020) and the annotations were added for the transcripts based on top hit. Moreover, the clean read of DEGs were subjected to classification and functional annotation by sequence comparison to gene ontology (GO)(Ashburner et al., 2000) , Swiss-Prot (Apweiler et al., 2004), Kyoto Encyclopedia of Genes and Genomics (KEGG)(Kanehisa et al., 2004) and Pfam (El-Gebali et al., 2019) databases. Along with that, the DEGs were submitted to iTAK-Plant Transcription factor & Protein Kinase Identifier and Classifier (Zheng et al., 2016) data base to identify differentially expression transcription factors from the different arsenic treated plants.

4.6. Development of transgenic *Arabidopsis* plants

4.6.1. Cloning of *AfACR3* and vector construction

Coding sequencing of *AfACR3* was amplified from cDNA of *Azolla* filiculoides using a primer set having BglII and BstEII restriction sites. The primer set was designed from the data obtained by analyzing by the transcriptome results and other already explored *ACR3* sequences of other organisms. Adapters also added during the cloning process through the primer designing to add restriction site of BglII at 5' end and BstEII 3' end. Subsequently, the amplified fragment was cloned in TA-cloning vector and sequence confirmation was done through Sanger sequencing. After sequence confirmation, coding sequencing of *AfACR3* was subcloned into the pCAMBIA1304 vector using BglII and BstEII restriction sites. Then bacterial transformation in *Escherichia coli* (DH5α) was done using heat shock method. Subsequently, PCR and restriction digestion confirmation was done. Finally, the Agrobacterium mediated transformation was done using the confirmed plasmid through freeze-thaw method.

4.6.2. Transformation, regeneration and analysis of over-expression line

To develop *AfACR3* overexpression *Arabidopsis thaliana* (Col-0) lines floral dip transformation method was followed. After transformation the plants were grown in a climate-controlled growth chamber having 16 hr light and 8 hr dark at 22 ℃. Subsequently, the seeds were set properly, and the seeds were harvested using Araharvester. To identify and select transformed plants, we used hygromycin as selection marker and then the selection was confirmed by PCR. Further, T1 and T2 lines were grown in growth chamber and the appropriate analysis were done using T2 lines with the wild type control. Plants were subjected to 45 μM As stress treatment for 6 days. Hydrogen peroxide (H_2O_2) content was checked as stress marker. Along with that chlorophyll fluorescence was measured in dark adapted plants with pocket PEA (Plant Efficiency Analyser, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) under dark conditions. For the determination of fluorescence induction an actinic light of 3500 $\mu molm^{-2}s^{-1}$ was used with peak wavelength of 627 nm.

4.7. Sub-cellular localization of *AfACR3*

To check the sub-cellular localization The coding region of *AfACR3* was amplified using gene-specific primers including the addition of the restriction site of BglII at 5' end and SpeI 3' end. The resulting PCR product was digested with BglII and SpeI, then cloned into the binary vector pCAMBIA1304, resulting in *Af*ACR3:GFP:1304. This vector was introduced into Agrobacterium tumefaciens strain LBA4404 using the freeze-thaw method.

For agroinfiltration agrobacterial strains containing *Af*ACR3:GFP:1304 vector were cultured in LB medium with appropriate antibiotics. The cultures were centrifuged, and the pellets were resuspended in an infiltration medium containing of 10 mM MES buffer, 10 mM $MgCl_2$, and 200 µM acetosyringone, adjusted to an OD600 of 0.8. These agrobacterial suspensions were infiltrated into the adaxial side of tobacco leaves using a needleless syringe. GFP expression in the infiltrated leaves was observed 48-96 hours post-infiltration (hpi) at various time intervals using fluorescent microscopy (Leica DMi8) (Sparkes et al., 2006).

5. Statistical analysis

The arsenic concentrations were calculated and expressed in µg/L. the data obtained from arsenic stress treatment and relative fold change in target gene expression were statistically evaluated by performing one-way ANOVA and the post-hoc analyses of the results were performed through Tukey's test at a significance level of $p \leq 0.05$ using IBM SPSS version 22 software.

6. Result and discussion

Objective 1: Characterization of arsenic tolerance mechanism of *Azolla filiculoides* under different arsenic (As) stress conditions.

The relative growth rates (RGR) of *Azolla* significantly declined after 6 days of As exposure, with a 91.4% reduction at 10 µM As and over 100% reduction at higher concentrations, indicating severe growth inhibition and possible necrosis (Fig.1a). Furthermore, the relative frond surface area (RFSA) decreased significantly with increasing As concentrations, except at 30 µM (Fig.1b). Chlorophyll content also declined with increasing As, notably a 63.6% reduction at 50 µM for chlorophyll a and significant reductions in chlorophyll b at higher concentrations (Fig.1c). Root traits were adversely affected by As stress. Root diameter increased by 16.21% at 40 µM As, while root length decreased significantly at higher concentrations (30, 40, and 50 µM). Root hair length showed minimal variation except at 40 µM, where it increased by 36.42% (Fig.1def). As accumulation in plant tissues increased significantly with higher As availability, with a 22-fold increase at 50 µM compared to 10 µM. Biochemical stress markers indicated substantial changes under As stress. Proline accumulation increased progressively with As concentration, reaching a 652% increase at 50 µM (Fig.2a). Peroxidase (POD) activity showed no significant change at 10 µM but increased markedly at higher concentrations, particularly

775% at 20 µM and 799% at 30 µM, with a decline at 40 µM (Fig.2b). Catalase (CAT) activity also increased significantly up to 30 µM, peaking at 20 µM with a 101% increase, but declined at 40 µM and 50 µM (Fig.2c). Hydrogen peroxide (H_2O_2) accumulation increased with rising As concentrations, with the highest increase of 546% at 20 µM (Fig.2d). Superoxide radical ($O_2^{\bullet-}$) accumulation followed a similar trend, with significant increases at higher As concentrations. Similar trend was observed after DAB staining of the plants where DAB is oxidized by H_2O_2 and forming dark brown patches in the *Azolla* fronds as well as in roots (Fig.3 a-f). The accumulation of superoxide radicals ($O_2^{\bullet-}$) inside the plant tissue was also observed with NBT staining by accumulation of blue coloured formazan compound deposition. We observed a steady incline the accumulation of superoxide radicals inside the plant tissue with increasing arsenic concentrations (Fig.3 g-l). Interestingly, when stained

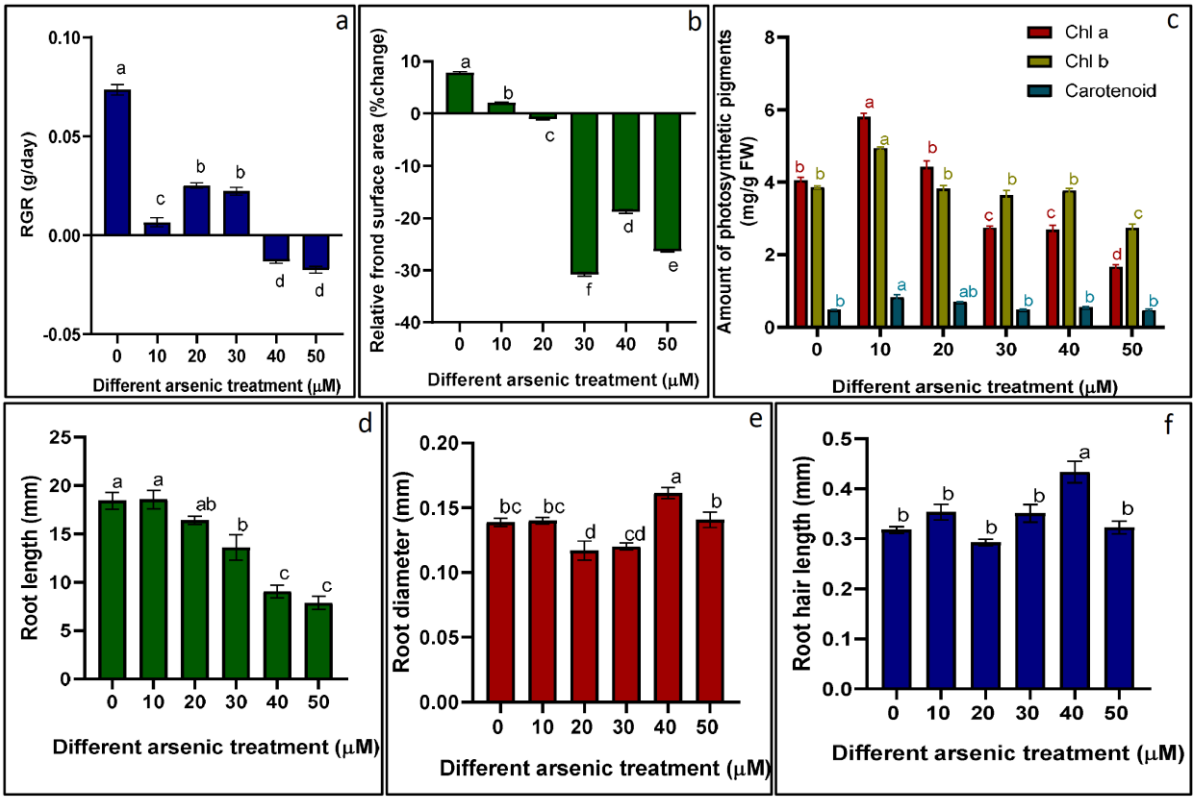

Figure 1: Effect of arsenic on (a) relative growth rate (RGR), (b) relative frond surface area and (c) estimate chlorophyll and carotenoid content; variation in (d) root length, (e) root diameter and (f) root hair length after 6 days of arsenic treatment. Data are means ± SE. Different letters indicate significant differences according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

with NBT we have observed a red-coloured deposition of superoxide radicals and partial plasmolysis in certain cells in the basal fronds of high arsenic treated plants (40 and 50 µM) (Fig.3 m-t). Previous study shows increased H_2O_2 and $O_2^{\bullet-}$ accumulation due to AsV

exposure in rice plants (Kumar et al., 2022). The chlorophyll fluorescence and OJIP transient analysis indicated changes in photosynthetic activity under As stress. At 50 µM, a decline in photochemical activity was observed, while at 20 µM, plants showed better photosynthetic performance compared to controls. The parameter Fv/Fm declined with increasing As, except at 20 µM (Fig4a). Other JIP test parameters, such as ABS/RC and TR_0/RC, increased under As stress, while ET_0/RC decreased, reflecting reduced electron transport flux (Fig4b). Previous study in soybean when subjected to arsenic stress shows similar effect on JIP test parameters (Vezza et al., 2022). Gene expression analysis revealed significant changes in arsenic-responsive genes. The multidrug and toxic compound extrusion (*MATE*) protein family is involved in secondary active transport responsible for expelling xenobiotic compounds (Kundu and Ganesan, 2020; Tiwari et al., 2014). The *MATE*1 gene expression

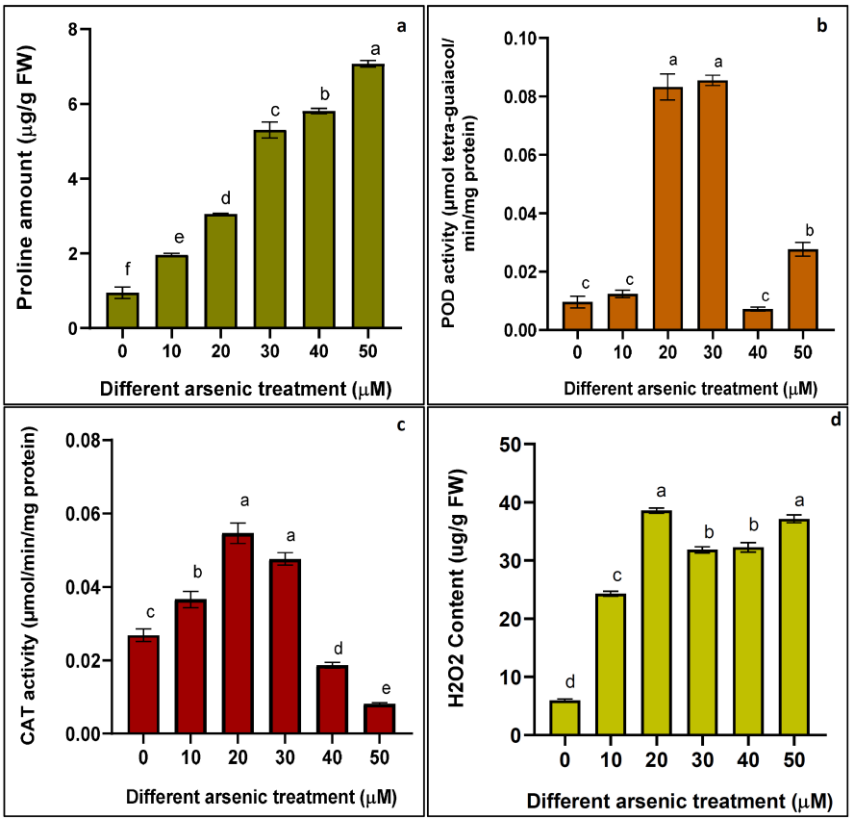

Figure 2: Effect on anti-oxidative enzymes activities, osmolyte and peroxides content subjected to different arsenic concentration after 6days. (a) Osmolyte (proline), (b) Peroxidase (POD), (c) Catalase (CAT) and (d) Hydrogen peroxide (H2O2). Data are means ± SE. Different letters indicate significant differences according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

decreased significantly at lower As concentrations but showed no significant changes at 30 µM and 40 µM. On the other hand, high arsenic content 1 (*HAC1*), which is responsible for encoding arsenate reductases; was identified as an arsenic responsive genes in previous

studies (Li et al., 2020; Shi et al., 2016). *HAC*1 expression increased significantly with higher As, peaking at 30 µM before declining at 50 µM. *CPK31* expression peaked at 10 µM As, decreased at 20 µM, and increased again at higher concentrations. *ACR3* expression increased significantly at 10 µM As but declined with higher concentrations, showing no notable differences among 20 µM, 30 µM, and 40 µM treatments, with no significant change at 50 µM (Fig5).

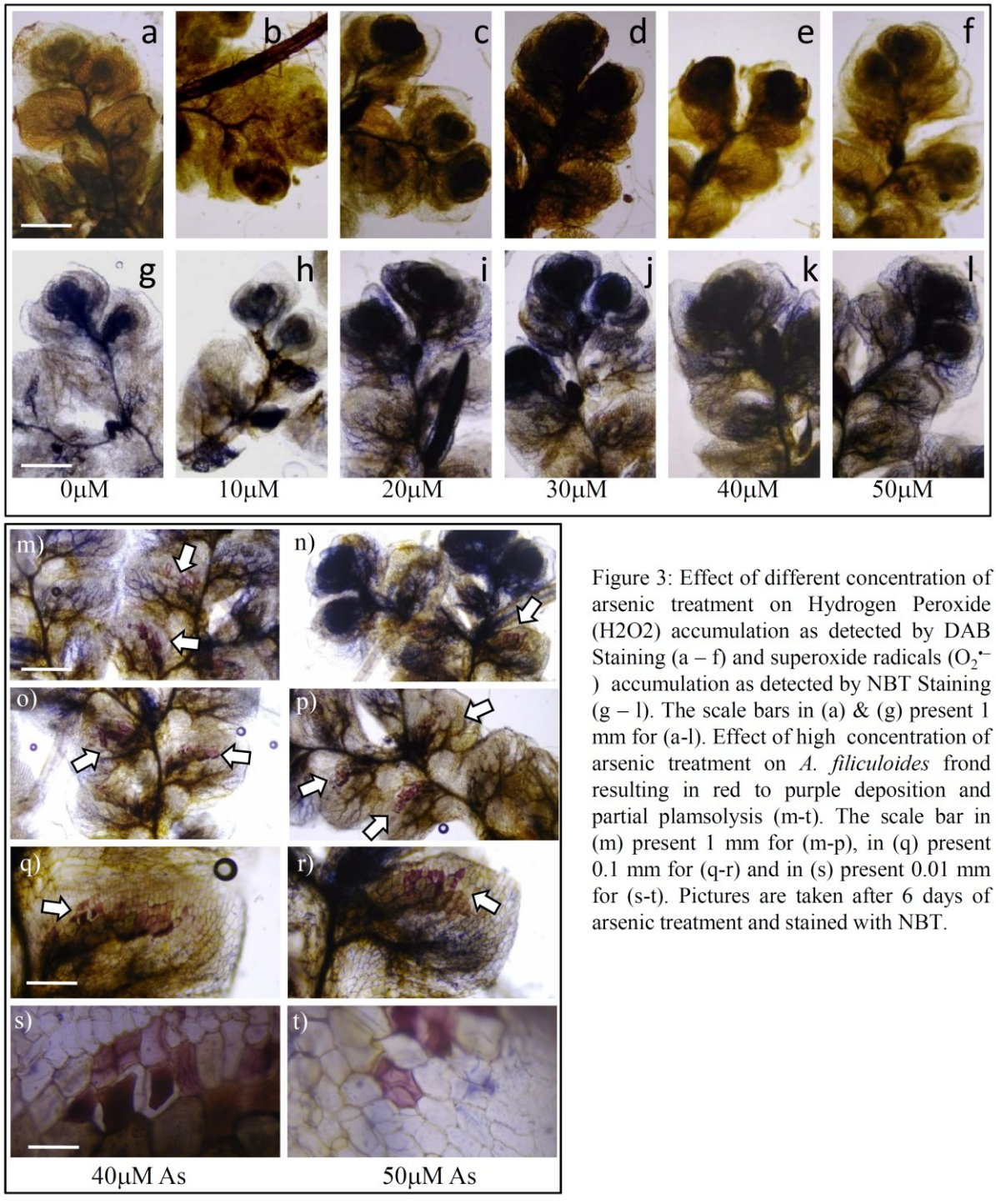

Figure 3: Effect of different concentration of arsenic treatment on Hydrogen Peroxide (H2O2) accumulation as detected by DAB Staining (a – f) and superoxide radicals ($O_2^{\bullet-}$) accumulation as detected by NBT Staining (g – l). The scale bars in (a) & (g) present 1 mm for (a-l). Effect of high concentration of arsenic treatment on *A. filiculoides* frond resulting in red to purple deposition and partial plamsolysis (m-t). The scale bar in (m) present 1 mm for (m-p), in (q) present 0.1 mm for (q-r) and in (s) present 0.01 mm for (s-t). Pictures are taken after 6 days of arsenic treatment and stained with NBT.

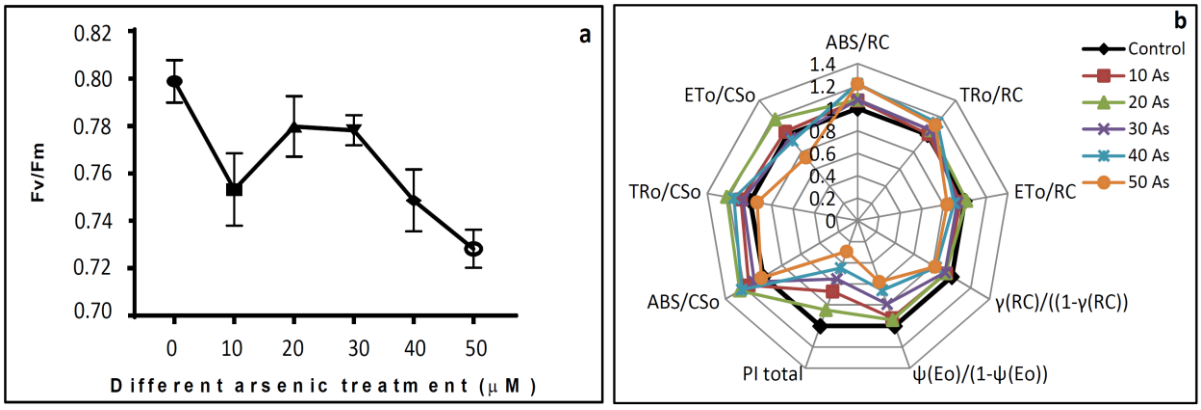

Figure 4: OJIP parameters of *A. filiculoides* plants after 6 days of arsenic stress treatment. a) F_V/F_M = ratio of variable fluorescence and maximum fluorescence, data are means ± SE; b) Radar plot with a series of parameters derived from JIP-test analyses of the experimental fluorescence OJIP transients, shows structure and function of photosynthetic apparatus under different arsenic stress conditions.

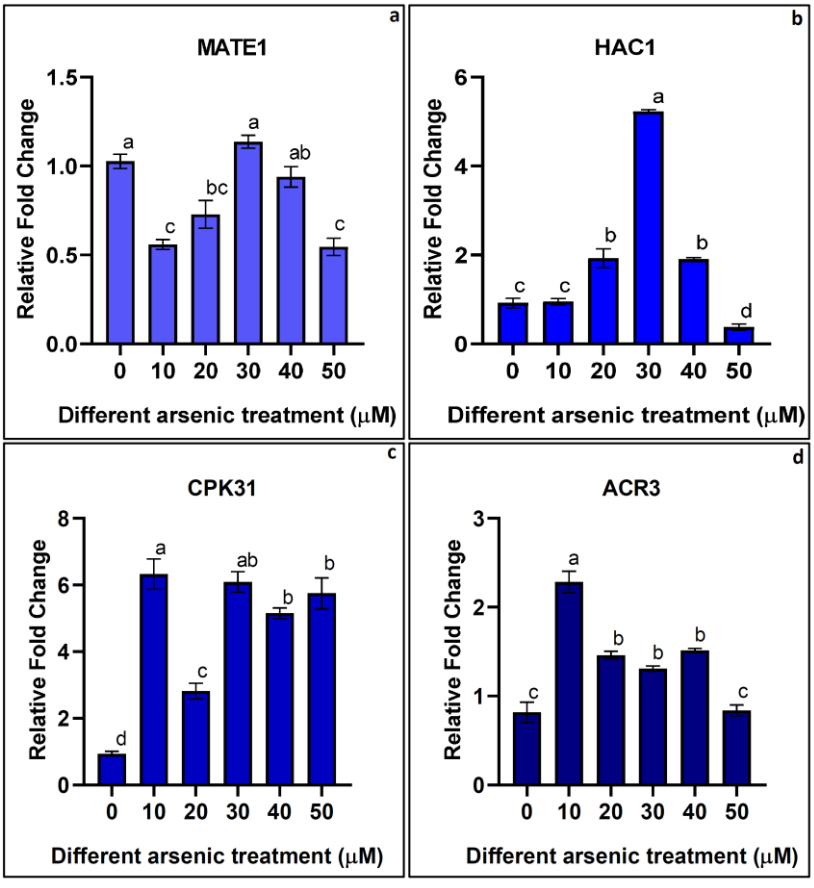

Figure 5: Effect of different arsenic (As) concentration treatment on various arsenic responsive genes in *Azolla* filiculoides Lam. Graphs showing qRT-PCR results represented in log fold change relative to reference expression of β-actin; (a) Multidrug and toxin extrusion 1 (MATE1), (b) High arsenic concentration 1 (HAC1), (c) Calcium dependent protein kinase 31 (CPK31), (d) Arsenic compounds resistance 3 (ACR3). Data are means ± SE. Different letters indicate significant differences according to one-way ANOVA followed by Tukey's multiple range test ($P < 0.05$).

Objective 2: Identification of the arsenic stress-responsive gene(s) by transcriptome assay from *Azolla filiculoides* treated with different arsenic stress conditions.

The transcriptomic analysis of *A. filiculoides* to arsenic stress through RNA-seq data from cultures treated with 0, 10, and 20 μM As revealed good sequencing quality, with clean reads used for de novo transcriptome assembly resulting in 775,191 genes. Subsequent clustering yielded 679,507 genes. A total of 7,179 differentially expressed genes (DEGs) were identified in response to arsenic stress (Fig6a,b). In the 10 μM treatment, 2,008 DEGs were up-regulated and 990 were down-regulated, while in the 20 μM treatment, 1,816 DEGs were up-regulated and 2,365 were down-regulated (Fig6c). The expression patterns of the top 1,000 significant DEGs were analyzed across all treatments (Fig6d). Among the DEGs, 532 were co-up-regulated, and 285 were co-down-regulated in both 10 μM and 20 μM treatments (Fig6e).

Functional annotation and classification of DEGs revealed enrichment in various cellular components and molecular functions associated with stress response and metabolism. KEGG pathway analysis highlighted pathways involved in protein modification, amino acid biosynthesis, and metabolism (Fig7). Proteolytic proteins have already been reported to be an arsenic stress marker in plants (Singh et al., 2022); our results also showed enrichment of a significant number of proteolytic protein DEGs in both of the arsenic stresses. Early light-inducible proteins are known to protect photosynthetic machinery but never been reported to be an arsenic responsive protein (Lee et al., 2020). Hierarchical clustering identified variably expressed DEGs, including Early light-inducible protein (*ELIP*) and Arsenic compound resistance 3 (*ACR3*) (Fig8a). Principal component analysis showed significant variation in expression between different arsenic concentrations (Fig8b). Transcription factor analysis revealed 75 DEGs significantly regulated by arsenic stress, involved in various biological processes including stress response and photosynthesis (Fig9a). Ethylene responsive transcription factors like ERF018 were down-regulated in response to arsenic (Fig9c); which showed that like *Arabidopsis thaliana*, in *A. filiculoides* also the arsenic responsive pathway activation partially regulated by ethylene (Singh et al., 2021). Similarly, several transcription factors were differentially co-expressed in response to arsenic stresses (Fig9b).

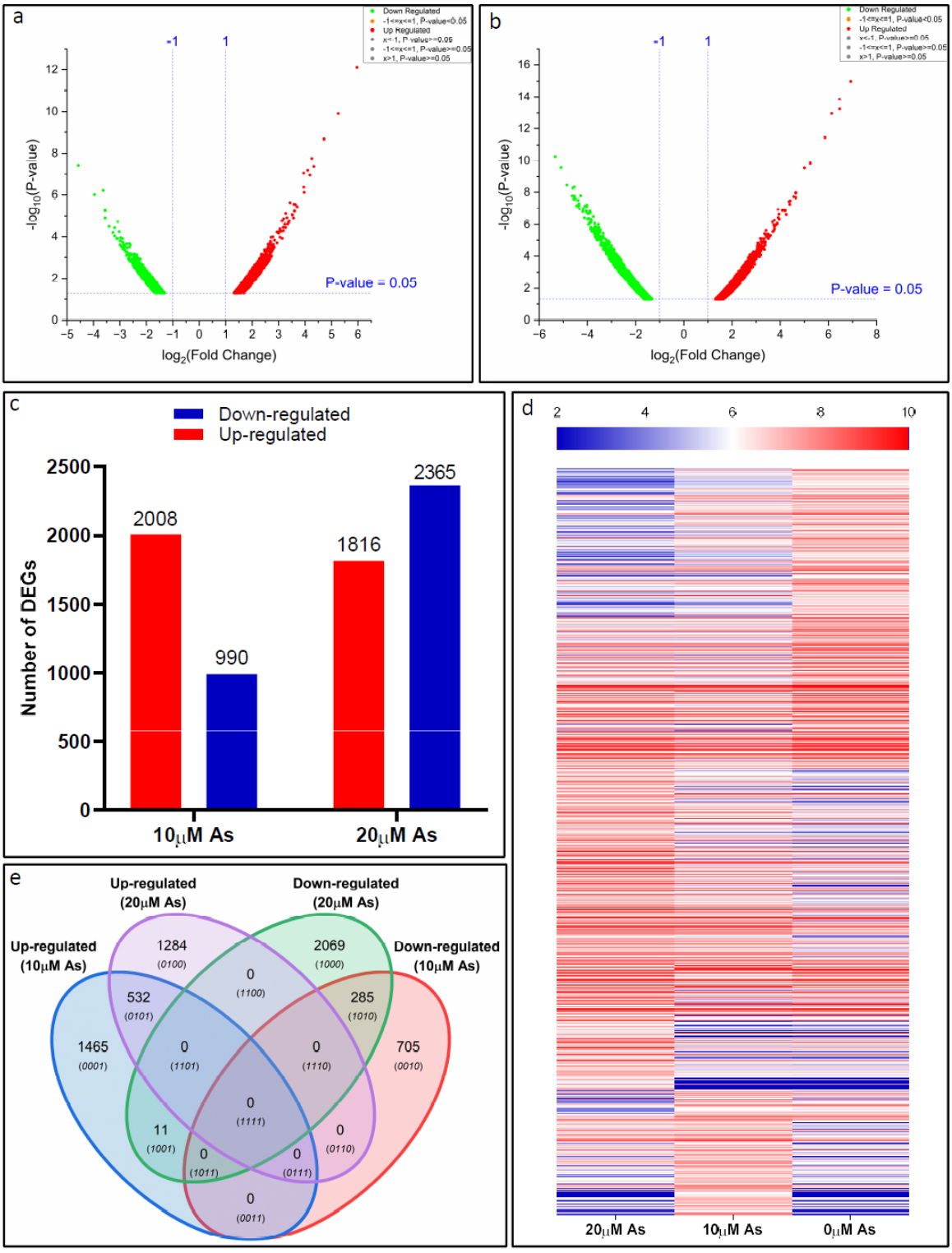

Figure 6: Overview of differentially expressed genes (DEGs) of *A. filiculoides* in response to different arsenic stresses. Significantly up and down regulated DEGs in (a) 10μM As, (b) 20μM As using threshold p-value < 0.05 and $\log_2$Fold-Change ≥ 1 or ≤ -1. (c) Number of up and down regulated DEGs in 10μM As and 20μM As treatment. (d) Heatmap showing normalized expression level of top1000 DEGs in 3 different arsenic stress treatments. (e) Venn diagram over lapping up and down regulation of significantly expressed DEGs in 10μM As and 20μM As concentration after 72hr of stress treatment.

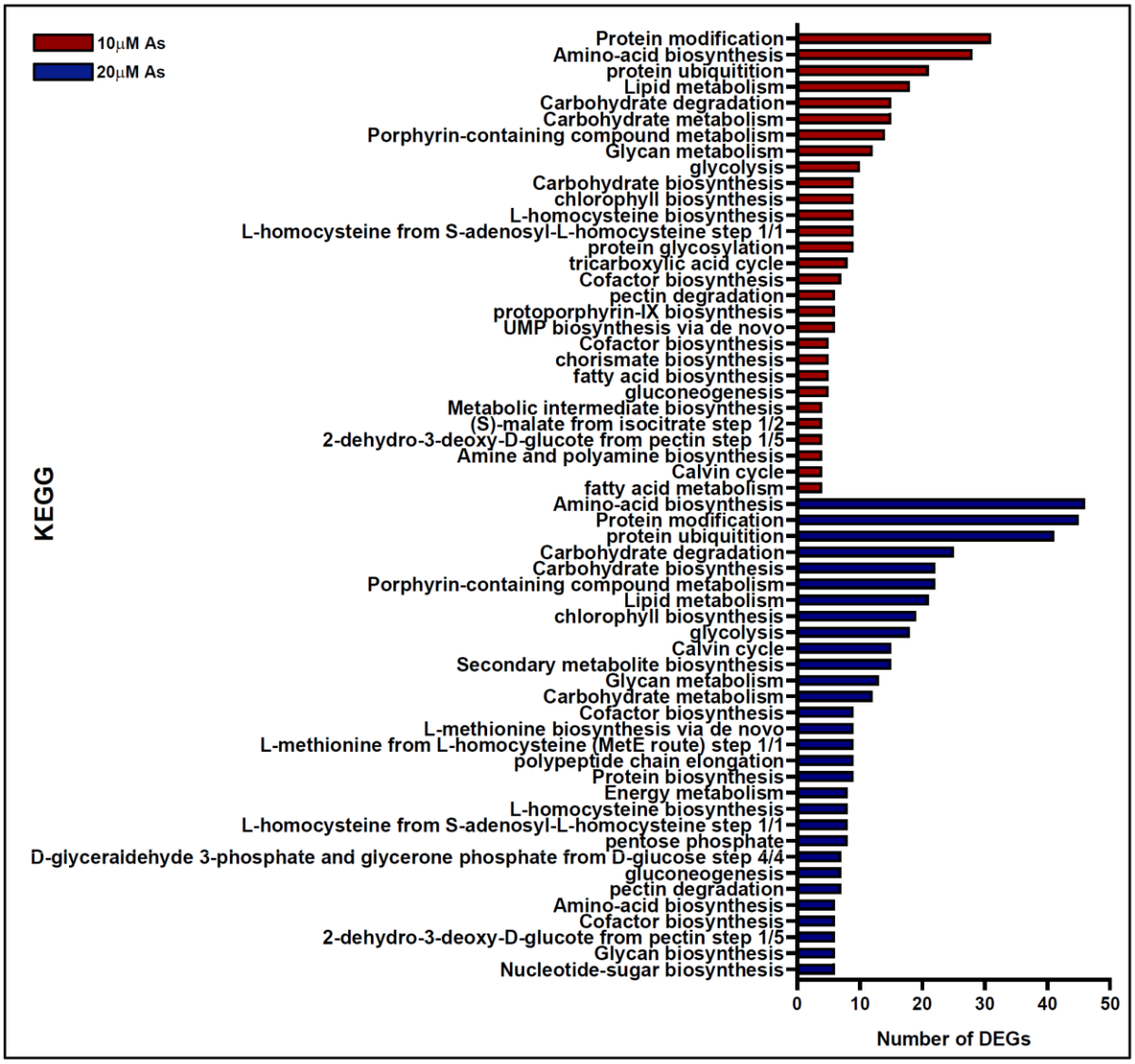

Figure 7 : KEGG pathway enrichment analysis of the DEGs in response various arsenic stress in *A. filiculoides* plants. KEGG analysis were done separately for the DEGs in10μM arsenic and 20μM arsenic after 72hrs of stress treatment.

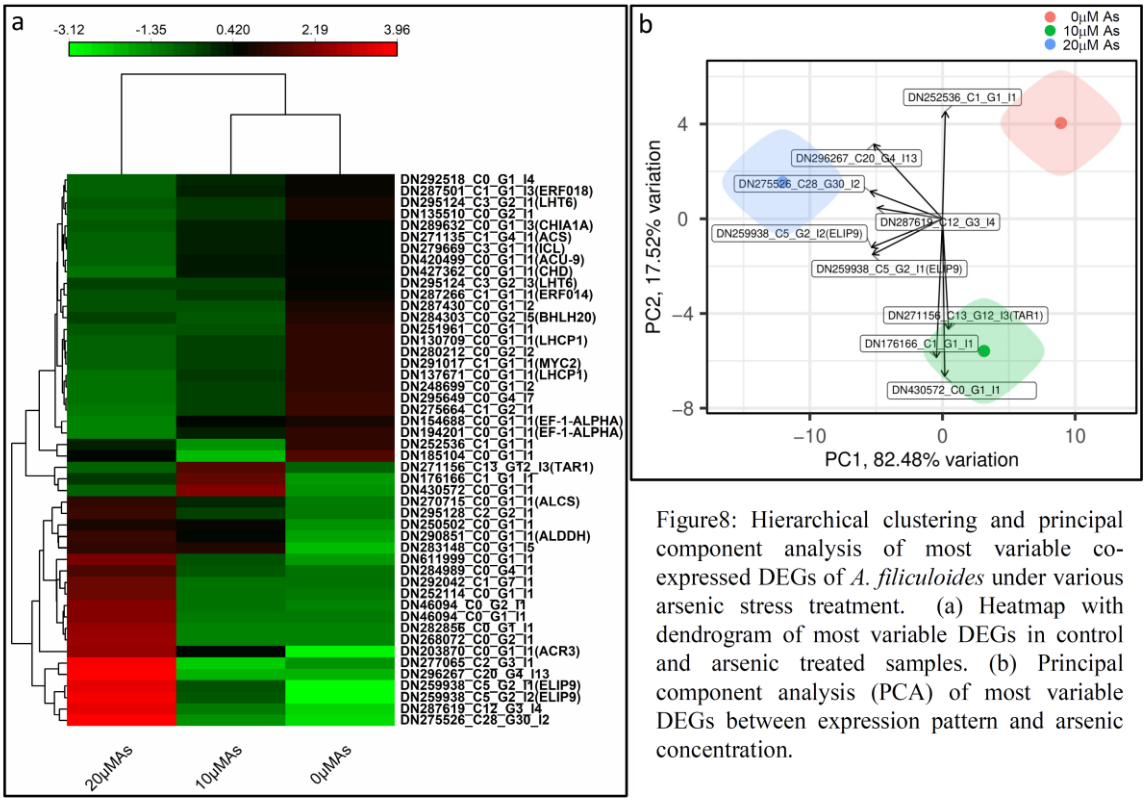

Figure8: Hierarchical clustering and principal component analysis of most variable co-expressed DEGs of *A. filiculoides* under various arsenic stress treatment. (a) Heatmap with dendrogram of most variable DEGs in control and arsenic treated samples. (b) Principal component analysis (PCA) of most variable DEGs between expression pattern and arsenic concentration.

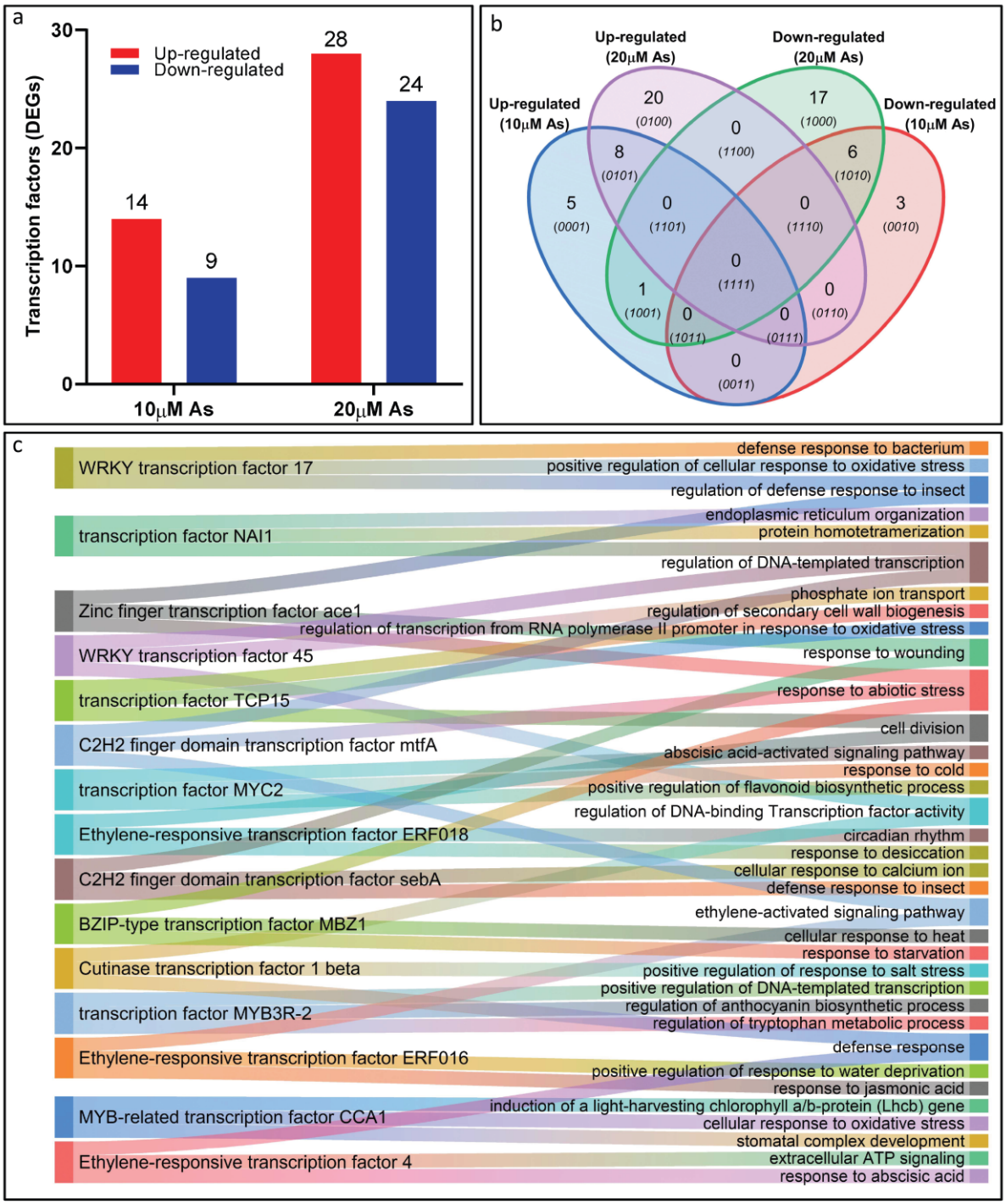

Figure9: Differentially expressed transcription factors of *A. filiculoides* in response to various arsenic stress treatments. (a) Numbers of up and down regulated transcription factor DEGs. (b) Venn diagram over lapping up and down regulation of significantly expressed transcription factors DEGs in 10μM As and 20μM As concentration after 72hr of stress treatment. (c) Sankey diagram showing co-expressed transcription factor DEGs in both arsenic stress treatments and their corresponding molecular functions.

Objective 3: Development of transgenic *Arabidopsis* plants with the key gene(s) which are responsible for arsenic stress-tolerance of *Azolla* plants.

Arsenic stress was given to T2 lines with the wild type control and transgenic control plants. We have observed a significant 23.42% increase of hydrogen peroxide (H_2O_2) accumulation in wild type arsenic stress treated plant when compared to wild type control plants. However, T2 line control and arsenic treated both sets of plant do not show any significant change in H_2O_2 accumulation when compared to wild type control plant (Fig10c). Similar results have been reported earlier suggesting hydrogen peroxide as a excellent As stress marker in *Arabidopsis thaliana* (Singh et al., 2021). Our result suggests that ACR3 plays a crucial role in arsenic stress management.

The chlorophyll fluorescence and OJIP transient analysis shows changes in photosynthetic activity under As stress. As treated wild type *Arabidopsis* plants shows decline in photochemical activity, while at As treated T2 plants showed better photosynthetic performance compared to controls (Fig11), which further depicts the importance of ACR3 in alleviating arsenic stress. The sub-cellular localization of *Af*ACR3:GFP in Tobacco plants revealed that the *Af*ACR3 is a plasma membrane protein (Fig12); ACR3 have never been reported in any aquatic plant or in any angiosperm either. The specific sub-cellular localization of AfACR3:GFP to the plasma membrane confirms that it is not a tonoplast protein. Therefore, it cannot be involved in the vacuolar sequestration of arsenic but only in arsenic efflux.

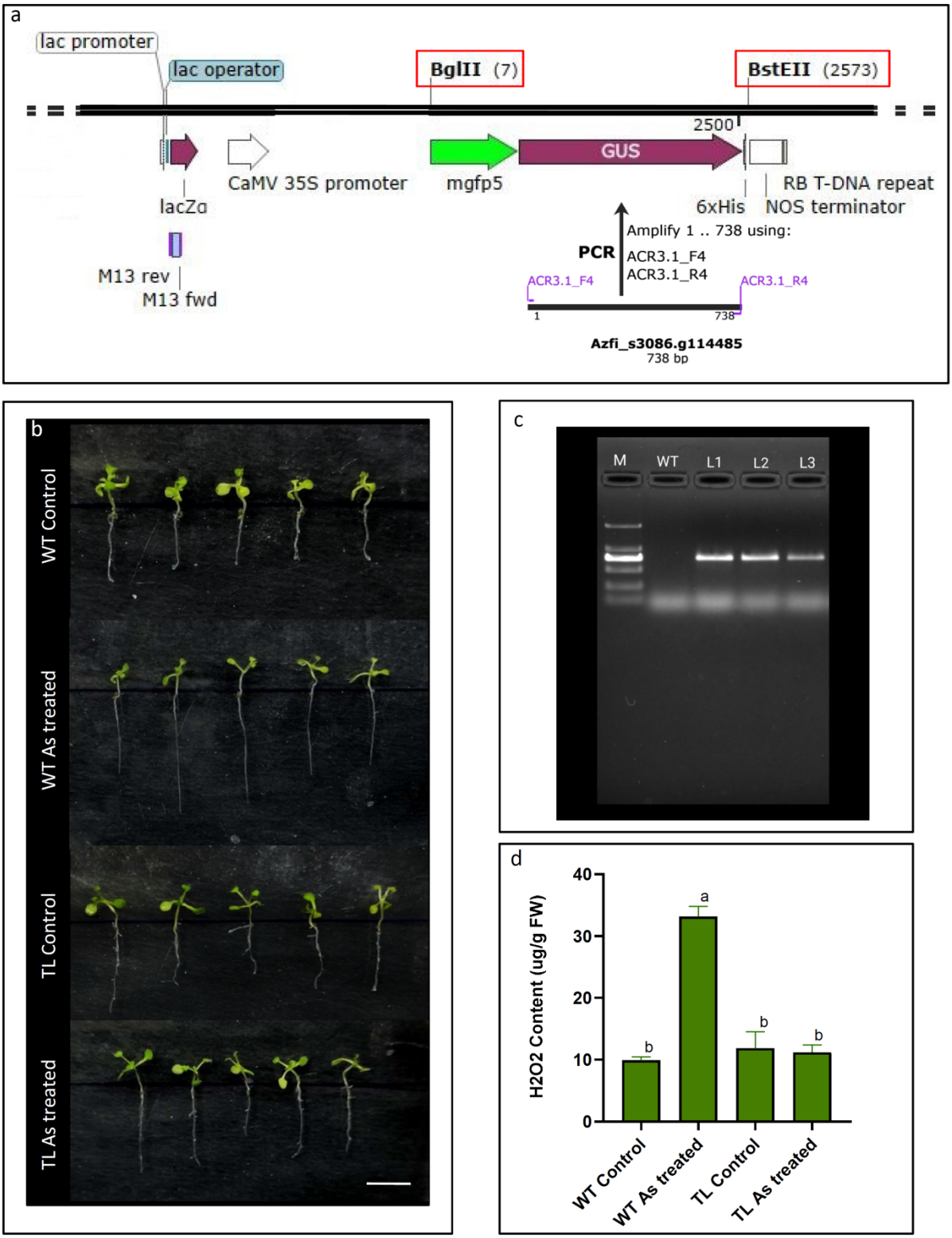

Figure 10: a) vector construct for *AfACR3* over-expression, b) Transgenic and Wild type *Arabidopsis* plant under arsenic stress condition, scale bar represents 10mm; c) PCR confirmation of *AfACR3* incorporation in *Arabidopsis thaliana;* d) Estimation of H2O2 from transgenic and wild type plants under As stress condition.

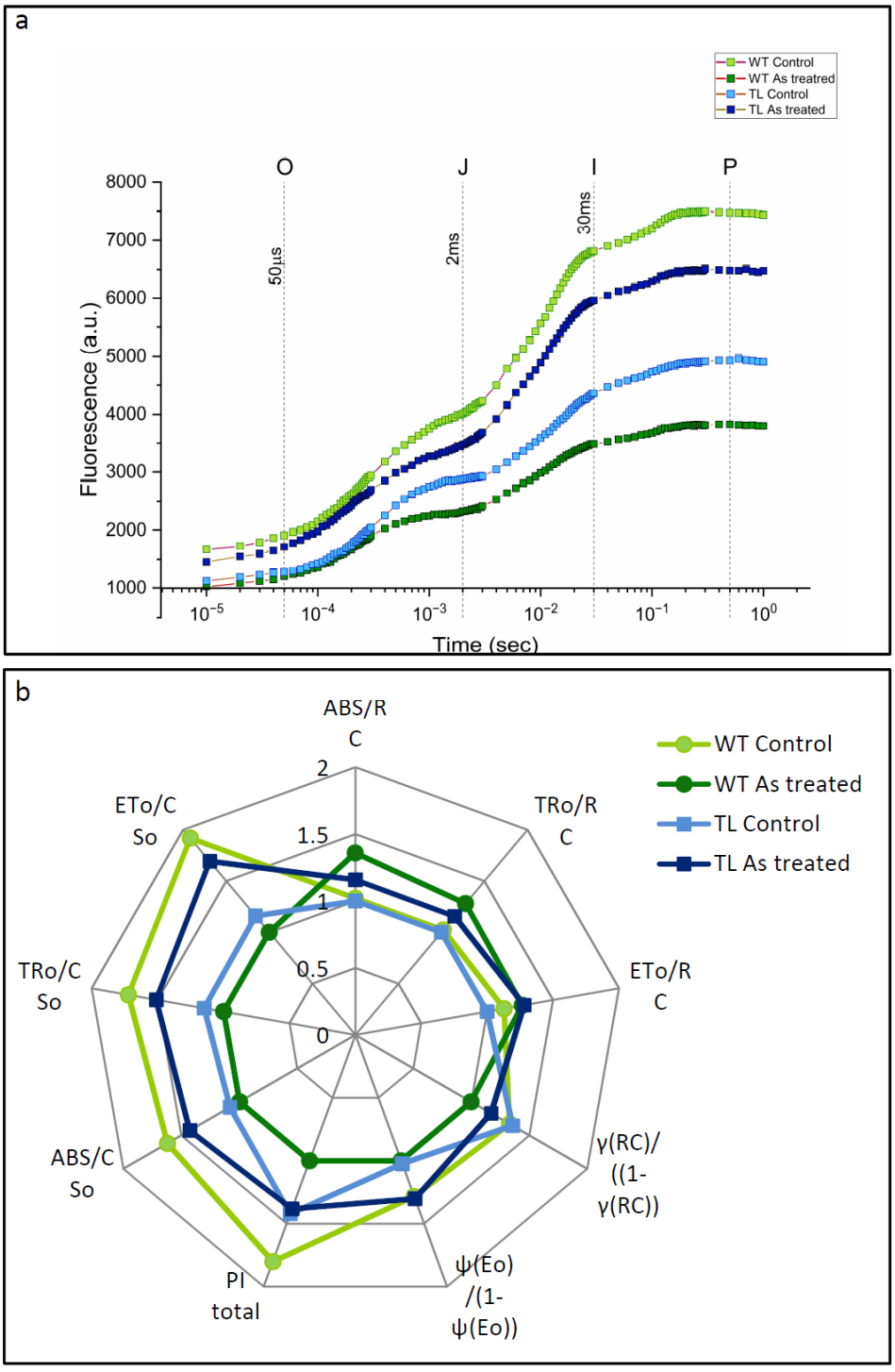

Figure 11:. a) Chlorophyll-a fluorescence kinetics of the OJIP curve of dark-adapted *Azolla* plants. Transient curves of each line represent the average of 5 measurements per treatment. b) Radar plot with a series of parameters derived from JIP-test analyses of the experimental fluorescence OJIP transients. ABS/RC = Apparent antenna size of an active PSII, Tro/RC = trapped energy flux per RC, Eto/RC = electron transport flux per RC, γ(RC)/((1-γ(RC)) = fraction of PS II chlorophyll-a molecules that function as RCs, ψ(Eo)/(1-ψ(Eo)) = efficiency with which a trapped excitaton transfers an electron to the photosynthetic ET chain further than QA−., PI_{total} =Performance index for energy conservation from photon absorbed by PSII to the reduction of PSI end acceptors; ABS/CSo = Absorption of photon flux per excited cross-section, TR_o/CSo = Maximum trapping per excited cross-section; ET_o/CSo = Electron transport flux per excited cross-section.

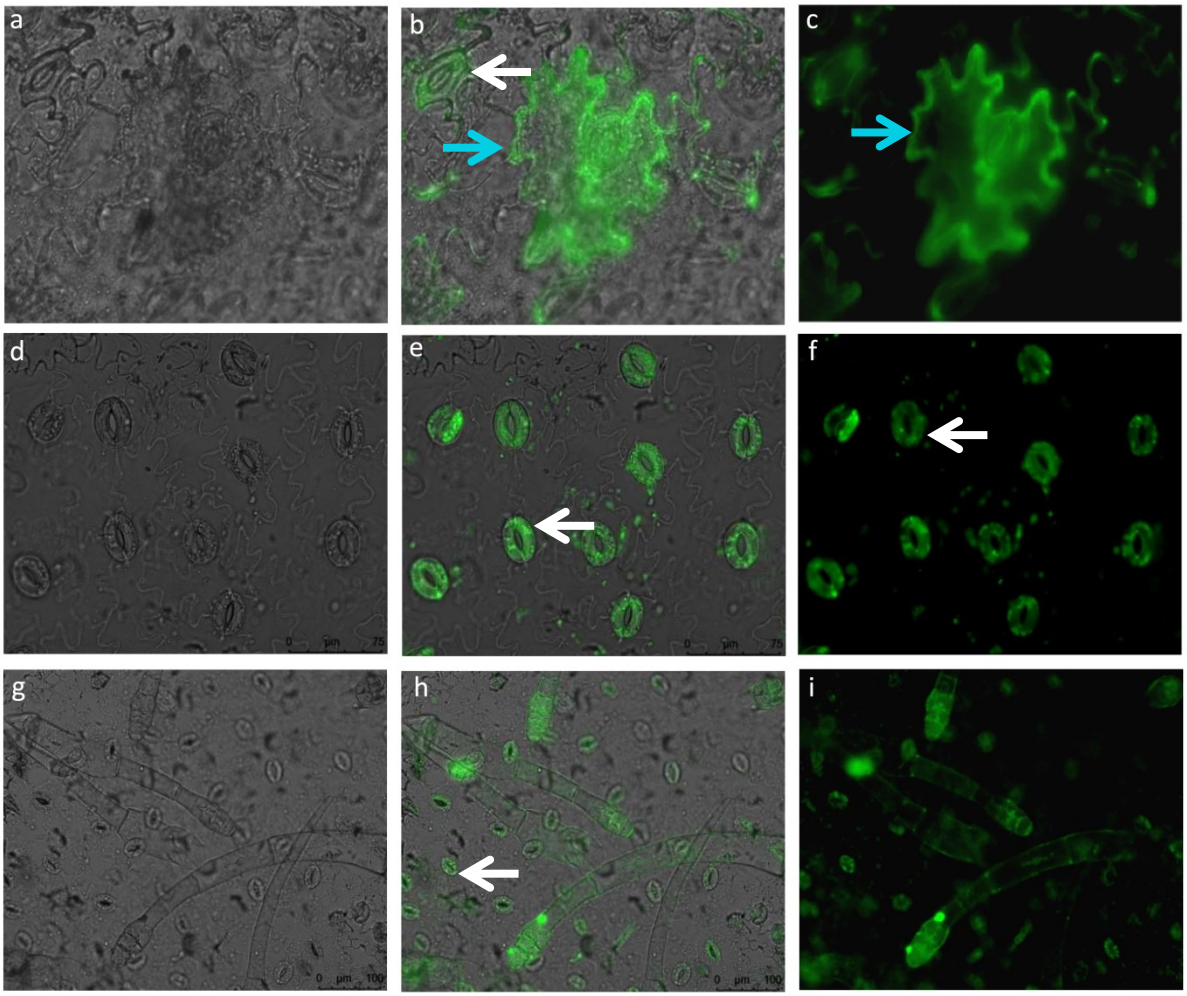

Figure 12 : Sub-cellular localization of AfACR3 by transient expression in tobacco leaves using Agroinfiltration. AfACR3:GFP:1304 expression fused AfACR3-GFP and cells were visualized at different planes. GFP expression of was recorded in the epidermal cells (Blue arrow) and guard cells (White arrow). GFP expression and localization was performed by the bright field images (a, d & g), fluorescence images (c, f & i) and overlay images of both bright field and GFP (b, e & h).

6. Conclusion

The identification of arsenic (As) hyperaccumulating aquatic plant species is vital for the remediation of contaminated aquatic environments. Our research has demonstrated that *Azolla* filiculoides possesses significant phytoremediation capabilities for arsenic, particularly at concentrations up to 30 μM. Notably, the nitrogen-fixing ability of *Azolla* was not significantly impaired under low to moderate arsenic stress, and the plants maintained healthy growth without stress phenotypes under these conditions. Additionally, on the physiological front the photosynthetic efficiency remained unaffected at arsenic levels up to 20 μM, as indicated by the F_v/F_m ratio and other OJIP parameters. Which indicates towards the presence of an arsenic tolerance mechanism in *Azolla filiculoides* plants which get optimum around moderate arsenic stress. Based on that data we have selected 10 and 20 μM arsenic treatment for our Transcriptome studies. Transcriptome analysis revealed that several transporters are regulated in *Azolla* in response to arsenic stress, suggesting mechanisms of tolerance and accumulation and highlighting the up-regulation of arsenic efflux and down-regulation of arsenic uptake as key tolerance mechanisms. Along with that several important genes are also revealed to be arsenic responsive which are previously associated with other abiotic stresses drought or redox homeostasis. And transcriptome data also indicated the presence of *ACR3* gene which was never identified in any aquatic plant. Then further work included making transgenic *Arabidopsis* plant with *AfACR3*, which revealed the significance of *AfACR3* in arsenic stress management. This comprehensive map of arsenic-responsive genes and their role in *Azolla filiculoides* provides a foundation for future studies toward understanding the arsenic tolerance mechanism and engineering arsenic resistant non-accumulative crop plants.

7. Significance of the study

Phytoremediation is one of the most economically and environmentally viable methods for arsenic removal from polluted sources. While arsenic-tolerant hyperaccumulator terrestrial plants have been explored for arsenic phytoremediation, aquatic plants have not been adequately studied for this purpose. In this study we have explored the arsenic phytoremediation properties of aquatic fern *Azolla filiculoides* along with the underlying key arsenic responsive genes for the better understanding of arsenic tolerant mechanism of aquatic plants and this will further broaden our horizon for the opportunity of exploiting environmentally friendly resources such as phytoremediation for arsenic removal. Along with that our work first ever reported the presence of arsenic efflux protein encoding gene *ACR3*

in aquatic plant which can further be used for genetically modifying crops to reduce arsenic accumulation in crops.

8. Future directions

Future research should focus on functional genomics, molecular, and physiological studies to fully elucidate the arsenic stress tolerance mechanisms in aquatic plants. Additionally, the role of *AfACR3* in arsenic efflux, as opposed to vacuolar sequestration, warrants further investigation, given its specific localization to the plasma membrane. Understanding these mechanisms will enhance the application of *Azolla* in phytoremediation and improve strategies for managing arsenic contamination in aquatic environments. Moreover, the collection of arsenic responsive genes revealed from our comparative transcriptomics study can be a steppingstone for further studies in exploring the arsenic metabolism and detoxification mechanisms is plants.

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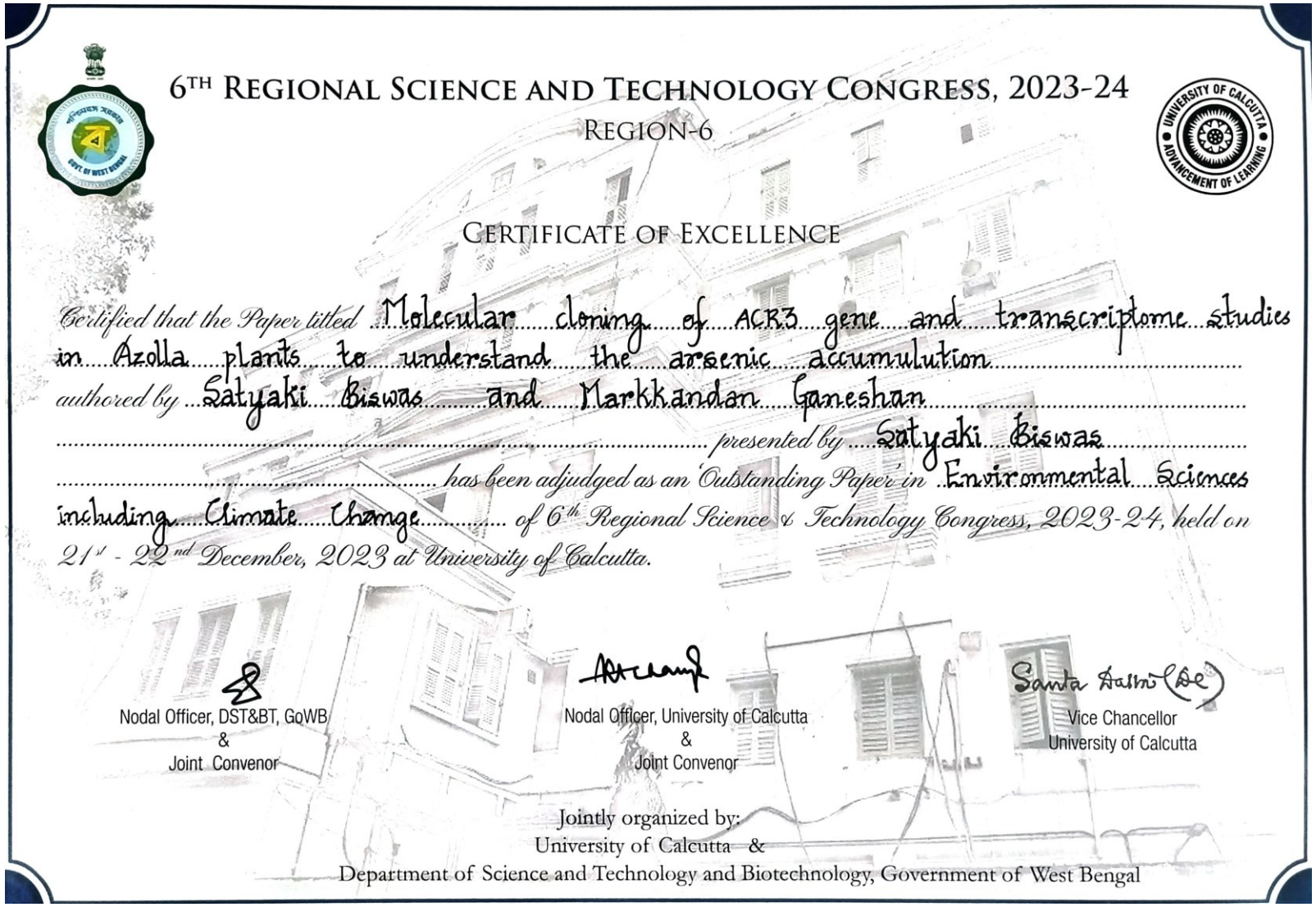

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Evaluation of arsenic phytoremediation potential in *Azolla filiculoides* Lam. plants under low pH stress conditions

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ABSTRACT

The *Azolla filiculoides* plants were challenged with different arsenic (As) concentration under low pH stress conditions. The growth rate and doubling time of the plants were severely affected by higher As treatments at pH 5.00 when compared with stress pH 4.75 treatments. Hence, pH 5.00 was considered for further studies. In 10–30 μM As treated cultures, after 6 days, the relative growth rate (RGR) of *Azolla* plants was significantly reduced and in higher concentration of As, the RGR was negatively regulated. The root trait parameters were also significantly affected by increasing concentrations of As. Further, photosynthetic performance indicators also show significant decline with increasing As stress. Overall, the plants treated with 40 and 50 μM of As displayed stress phenotypes like negative RGR, reduced doubling time and root growth, browning of leaves and root withering. The total proline, H_2O_2, POD and Catalase activities were significantly affected by As treatments. Meantime, 30 μM of As treated cultures displayed 15 μg/g/Fw As accumulation and moderate growth rate. Thus, the *Azolla* plants are suitable for the phytoremediation of As (up to 30 μM concentration) in the aquatic environment under low pH conditions (5.00). Furthermore, the transcriptome studies on revealed that the importance of positively regulated transporters like ACR3, AceTr family, ABC transporter super family in As (10 μM) stress tolerance, uptake and accumulation. The transporters like CPA1, sugar transporters, PiT were highly down-regulated. Further, expression analysis showed that the *MATE1*, *CIP31*, *HAC1* and *ACR3* were highly altered during the As stress conditions.

1. Introduction

Arsenic (As) is one of the most abundant metalloids which poses a great threat to humankind due to its abundance and toxic nature; arsenic exposure can cause a plethora of potentially fatal diseases including arsenicosis, neurodegenerative diseases, cardiovascular diseases and cancer in human, along with that arsenic stress is one of the major abiotic stresses faced by crop plants (Argos et al., 2010; Guha and Dasgupta, 2011; Milton et al., 2005; Podgorski and Berg, 2020; Smith et al., 2002; Taylor and Mukherjee, 2003). In spite of the efforts of marking and avoiding of ground water sources arsenic is still finding its way to human system primarily by crop plants (Jian et al., 2008). Thus, it is utterly important to try to understand the process of accumulation, assimilation and remediation of arsenic. In nature, there are two major inorganic forms of arsenic, arsenate (AsV) and arsenite (AsIII); among these arsenate (AsV) is more bio-available due to its structural similarity with inorganic phosphate (Mitra et al., 2017; Tripathi et al., 2007). In ground water both +3 and + 5 oxidation states of arsenic is commonly present and considered toxic but the +3 state or Arsenite (AsIII) form remains unassociated like arsenous acid (H_3AsO_3) in a wide range of pH and enters in plants through *NODULINE LIKE INTRINSIC PROTEINS* (NIPs) (Biswas and Ganesan, 2024; Gregus et al., 2009; Mishra et al., 2021). Arsenic toxicity, in case of plants predominantly falls on the speciation of arsenic, among different arsenic species, the inorganic arsenic species i.e., AsV and AsIII are the most toxic ones (Li et al., 2016).

Arsenic enters the plant systems in form of arsenate (AsV) through the phosphate transporters present in the root but along with that a significant portion of arsenic uptake take place via aquaporins and other non-specific transporters (Srivastava et al., 2007). After entering the plant system Major part of the arsenate (AsV) is converted to arsenite (AsIII) by arsenate reductases; which mainly subjected to phytochelation or extrusion (Biswas and Ganesan, 2024). So, either it can be chelated within the plant cell to a non-toxic macromolecule or to be extruded from the plant altogether. The chelation of arsenic in plant cell dominated by glutathione (GSH), metalothiones (MTs) (Mishra et al.,

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REVIEW ARTICLE

Current perspectives of ACR3 (arsenite efflux system) toward the reduction of arsenic accumulation in plants

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Abstract
Arsenic is one of the most detrimental heavy metals on the planet due to its bio-accumulative and carcinogenic nature. In addition to humans and animals, it also has a hazardous foot print on agriculture. Arsenic in its inorganic form is readily bio-available from water sources and taken up by the crop plants through phosphate transporters due to its structural similarity with inorganic phosphate. Bio-accumulation of arsenic in crop plants can lead to an arsenic-contaminated food chain and amplify manifold the hazardous effects of arsenic pollution. Apart from phosphate transporters, different aquaporins like Nodulin26-like intrinsic proteins, plasma membrane intrinsic proteins, tonoplast intrinsic proteins, and vacuolar transporters are responsible for arsenic uptake and accumulation in plants. To withstand a higher concentration of arsenic, bacterial and fungal systems use ArsB and Acr3 efflux transporter proteins, respectively, to pump out the arsenic from the cell. In plants, Acr3 (Arsenic Compound Resistance 3) efflux protein has been reported only in Pteridophytes to efflux arsenic from cells. In this review, we have discussed different transporters involved with arsenic uptake, translocation, sequestration, and efflux with special emphasis on Acr3. We have also explored the transgenic approaches performed with ACR3 to reduce arsenic accumulation in plants and discussed the future prospects of reducing arsenic from the food chain by lowering arsenic accumulation altogether from crop plants.

Keywords Phytoremediation · Heavy metal · Transgenes · Plants · Toxicity · Genetic regulation

Introduction

Arsenic (As) is one of the most toxic heavy metal pollutants which affects plants, animals, and humans all over the globe; and impose a threat to mankind as it can contaminate the groundwater supply and irrigation system of many countries (Guha and Dasgupta 2011; Milton et al. 2005; Smith et al. 2002; Taylor and Mukherjee 2003). Apart from being one of the most abundant elements of earth, the sources of arsenic can be diverse. In recent times, arsenic has been used in pesticides which are a potent source to pollute water sources. Prolong arsenic exposure can induce neurodegenerative diseases, cardiovascular complications, and can also appear as a carcinogenic in certain cases (Li et al. 2017; Argos et al. 2010; Chen et al. 2011). There are two major ways of arsenic exposure which include use of arsenic-contaminated water and crops. Another potential way of exposure of arsenic is from livestock and other products derived from the vegetation of arsenic-contaminated areas. Apart from the use of direct contaminated water, the arsenic in crops and livestock end up in the food chain, which is due to the arsenic uptake by the crop plants. Arsenic in plant systems translocates through phosphate transporter proteins and accumulates in shoot and root tissue; in case of crop plants like rice, arsenic accumulates in shoot and grain through silicon transporters (Jian et al. 2008).

There are different valence states in which arsenic can exist in nature; and arsenic salts have a wide range of solubility which firmly rely on the pH condition and ionic environment (Tripathi et al. 2007). Two major forms of inorganic arsenic are abundant in environment arsenate (AsV) and arsenite (AsIII); among them, arsenate (AsV) is dominant in oxygenated condition but arsenite (AsIII) is in stable form in reducing environment (Mitra et al. 2017). However, organic forms of arsenic like monomethylarsonic acid (MMA) and dimethyl arsenic acid (DMA) also exist in nature but they are not abundant as

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